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Recombinant bovine soluble CD14 sensitizes the mammary gland to lipopolysaccharide

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Abstract

Standard therapies including administration of potent antibiotics, aggressive fluid resuscitation and metabolic support have not been successful in relieving symptoms and reducing mortality associated with acute coliform mastitis. It is important to understand the pathophysiological response of the mammary gland to coliform infections when designing preventive or therapeutic regimens for controlling coliform mastitis. Our laboratory has previously shown that macrophages and polymorphonuclear neutrophils in milk express CD14 on their cell surface. In this study, we found that soluble CD14 (sCD14) is present in milk whey as a 46 kDa protein reacted with anti-ovine CD14 antibody. Additional functional studies found that: (1) under serum-free condition, complexes of LPS-recombinant bovine soluble CD14 (rbosCD14) induced activation of mammary ductal epithelial cells (as measured by changes in interleukin-8 (IL-8) mRNA level by competitive RT–PCR) at low concentrations of LPS after 6 or 24 h incubation (1–1000 ng/ml), whereas LPS alone did not induce activation of mammary ductal epithelial cells at the same concentrations, and (2) intramammary injection of low concentrations of LPS did not increase concentration of leukocytes in milk. In contrast, LPS–rbosCD14 complex containing the same concentration of LPS increased the concentration of leukocytes in the injected mammary gland at 12 and 24 h post-injection. These results indicate that rbosCD14 sensitizes mammary epithelial cells to low concentrations of LPS in vitro and in vivo. Endogenous sCD14 in milk may be important in initiating host responses to Gram-negative bacterial infections.

Keywords: Mastitis; Coliforms; Interleukin-8; CD14

1. Introduction

Mastitis is the most costly disease in the dairy industry, with economic losses of approximately $ 1.8 billion annually in the United States (National Mastitis Council, 1996, p. 2). Mastitis results in decreased milk production, increased veterinary cost and early culling or death of animals. Coliform mastitis is the most prevalent form of clinical mastitis, with infection by *Escherichia coli* being the most frequent. Approximately 80% of all intramammary infections by coliform bacteria will result in clinical mastitis, and about 10% will become peracute with the sudden onset of severe clinical symptoms of endotoxin shock (National Mastitis Council, 1996, p. 12). Because coliforms are present in the cow's environment, they

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; LBP, LPS-binding protein; MSCC, milk somatic cell count; NC, nitrocellulose; rbosCD14, recombinant bovine soluble CD14; sCD14, soluble CD14; mCD14, membrane CD14

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cannot be eradicated on a practical basis. Conventional herd management practices such as pre- and post-milking teat dipping and dry cow antibiotic therapy are unable to reduce the incidence of new infections. Coliform mastitis will exist as an animal health problem even in well-managed herds. In addition, antibiotic treatment, extensive fluid supplementation and metabolic support are not effective in relieving symptoms associated with clinical coliform mastitis (National Mastitis Council, 1996, pp. 11–12). Understanding the pathophysiological response of the mammary gland to coliform infections is critical to design novel preventive and therapeutic regimens for clinical coliform mastitis.

It was postulated that microbial products, often present in adjuvants, act on the innate immune system to elicit signals for activation of the adaptive immune system (Janeway, 1989; Medzhitov and Janeway, 1997). Kinetic studies of experimental coliform mastitis induced by the intramammary injection of *E. coli* showed that an inflammatory response will not be initiated until bacterial growth reaches a certain level (Shuster et al., 1995, 1996). The uncontrolled bacterial growth results in a buildup in the concentration of microbial products that can be recognized by the host as a danger signal for the presence of a bacterial infection. It is conceivable that this signal is comprised of a conserved group of molecules across bacterial groups, and the host processes a sensitive machinery to detect this danger signal after it exceeds a certain threshold. Lipopolysaccharide is one of the best characterized candidates of a danger signal because LPS is a component of the outer membrane of all Gram-negative bacteria, and is released by actively growing, damaged and dead bacteria (Petsch and Anspach, 2000). The toxicity of LPS is attributed to lipid A, a conserved domain of LPS. In addition, mammals process a CD14-dependent pathway to detect subpicomolar concentrations of LPS that activate host cells to mount an inflammatory response for clearance of bacteria (Wright et al., 1990; Dentener et al., 1993; Ulevitch and Tobias, 1999).

Two forms of CD14 exist. Membrane bound CD14 (mCD14) is present on the cell surface of monocytes, macrophages and polymorphonuclear neutrophils (PMNs), and mediates activation of those phagocytes by low concentrations of LPS in the presence of LPS-binding protein (LBP). Soluble CD14 (sCD14) is present in serum/plasma, and urine of nephrotic patients (Maliszewski et al., 1985; Bazil et al., 1986; Haziot et al., 1988), and mediates activation of cells not bearing mCD14 including epithelial cells and endothelial cells (Frey et al., 1992; Arditi et al., 1993; Pugin et al., 1993; Read et al., 1993). Macrophages are the predominant cell type in milk from uninfected bovine mammary glands. Bovine macrophages and PMN in milk express mCD14 on their cell surface (Paape et al., 1996), and are potential sources of sCD14. In later studies by our laboratory (Wang et al., 1997), we detected the presence of sCD14 in bovine milk. Recently, Filipp et al. (2001) reported sCD14 in both bovine colostrum and milk, and that sCD14 in both the native and recombinant form induces murine and human B cell growth and differentiation. However, the functional role of bovine sCD14 in milk, especially in the pathogenesis of coliform mastitis, is undefined. Because LBP or LBP-like proteins have not been identified in milk, the role of mCD14 in activation of milk macrophages by LPS prior to leakage of plasma components into the mammary gland is unknown. We hypothesized that sCD14 in milk mediates activation of mammary ductal epithelial cells by low concentrations of LPS. This activation may contribute to udder swelling and changes in vascular and mammary epithelium permeability, which are the first two clinical signs observed after experimental coliform infections (Shuster et al., 1995, 1996).

In this study we determined that: (1) rbosCD14, generated by a baculovirus/insect cell expression system, enhanced the sensitivity of mammary ductal epithelial cells to LPS in vitro, as measured by changes in transcription of IL-8 mRNA, and (2) intramammary injection of rbosCD14 sensitizes the mammary gland to LPS.

2. Materials and methods

2.1. Preparation of milk whey

Milk was collected aseptically from four clinically normal mid-lactation Holstein cows. Mammary glands were determined free from intramammary infection after culturing milk on blood agar plates. Milk fat was removed after centrifugation at $1000 \times g$ for

30 min at 4 °C. Whey was prepared by centrifuging the skimmed milk at 10 000 × *g* for 30 min at 4 °C. The whey was stored at −70 °C until Western blot analysis.

2.2. Expression and purification of rbosCD14

Total RNA from the lung of a Holstein cow was isolated using Tri-reagent (Sigma, St Louis, MO) according to the manufacturer's instructions. First strand cDNA was synthesized using Superscript RT II system (GIBCO-BRL Life Technologies, Gaithersburg, MD) with an oligo(dT) primer. The cDNA coding the N-terminal 358 amino acids of bovine CD14 was PCR amplified using the sense primer boCD14F1 (5′-AAAGAATTCATGGTGTGCGTG-CCCTACC-3′), and the antisense primer boCD14R2 (5′-AAAAAGCTTACGCGAAGCCTCGG-GCTCC-TTGAAAG-3′). The boCD14F1 and boCD14R1 primers contained *Eco*RI and *Nco*I restriction sites, respectively, which permitted digestion of the PCR fragment with the cognate enzyme and subcloning into modified pBlueBac 4.5 vector (Invitrogen, Carlsbad, CA). The pBlueBac 4.5 was modified by replacing sequence between *Nco*I and *Hin*dIII sites with a six histidine tag. One clone designated clone 36 was confirmed to contain the N-terminal 1–358 amino acids of bovine CD14 by automated sequencing.

A recombinant virus containing the N-terminal 1–358 amino acids of bovine CD14 (rbosCD14) was generated by co-transfection of insect sf-9 cells with clone 36 and linear Bac-N-Blue DNA (Invitrogen) according to manufacturer's instructions. Insect high five cells at a density of 2×10^6 cells/ml were infected with the recombinant virus at a multiplicity of infection of 10. To determine the time course of expression of rbosCD14, culture supernatant (1 ml) was collected at 0 (infection), +24, +48, +72, +96 and +120 h post-infection, and assayed for the presence and amount of rbosCD14 by Western blot.

Purification of rbosCD14 from the culture supernatant was performed according to Tapping and Tobias (1997). Briefly, the cell culture was collected at 96 h post-infection, and centrifuged (100 × *g* for 5 min). The supernatant was further centrifuged (6000 × *g* for 10 min) and filtered through a 0.22 mm filter. The supernatant was dialyzed (4 °C, overnight) against 0.0132 M PBS, pH 7.4 and buffer A (100 mM sodium phosphate, 300 mM sodium chloride, pH 8.0) in a dialysis tube with a molecular weight cutoff at 10–12 kDa (Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed culture supernatant was incubated with Ni-NTA superflow agarose beads (Qiagen, Valencia, CA) in the presence of 10 mM imidazole at room temperature for 3 h on an orbital shaker set at 150 rpm. The Ni-NTA beads were packed into XK16/20 column (Amersham Pharmacia, Pitscataway, NJ). The column was connected to an FPLC (Amersham Pharmacia), washed with a ×10 column volume of buffer A containing 25 mM imidazole, and rbosCD14 was eluted with 100 mM of imidazole in buffer A. Fractions (4 ml) were collected using a FRAC-200 fraction collector (Amersham Pharmacia) and analyzed for CD14 by SDS–PAGE and Western blot.

2.3. Western blot to determine the presence of sCD14

The whey or culture supernatant was combined with an equal volume of non-reducing 2 × Lammeli buffer and heated at 70 °C for 10 min. Proteins were separated on a 10% resolving Tri–glycine gel with 4% stacking gel, and transferred onto a nitrocellulose (NC, BioRad, Hercules, CA) membrane. After blocking in 0.0132 M PBS containing 1% BSA (Sigma) and 0.01% Tween-20 (Sigma), the NC membrane was probed with anti-ovine CD14 mAb (Serotec, Raleigh, NC) or anti-tetra-his-mAb (Qiagen, Valencia, CA) and then alkaline phosphase conjugated goat-anti-mouse IgG (Kirkegaard & Perry Labs, Gaithersburg, MD). The NC membrane was developed using a BCIP/NBT kit (Kirkegaard & Perry Labs).

2.4. Mammary ductal epithelial cell culture

Epithelial cells from the ductal region of the mammary gland of a lactating cow was kindly provided by Dr. Albert Guidry (Cifrian et al., 1994). Cells were cultured on collagen-coated 60 mm dishes (Beckman Dickinson, San Diego, CA) in culture medium containing 40% RPMI 1640 (Hyclone, Logan, UT), 40% Dulbecco's modified Eagle's medium (DMEM; Hyclone), 10% FBS (Hyclone), 1% antibiotic–antimycotic solution (Life Technology, Gathersburg, MD), 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Hyclone), 40 mM HEPES buffer

(Hyclone), bovine insulin (5 μg/ml, Sigma), hydrocortisone (1 μg/ml; Sigma) and bovine prolactin (1 μg/ml; courtesy of Dr. Anthony Capuco, USDA, Beltsville, MD).

2.5. Stimulation of ductal epithelial cells with LPS

A 6-well plate was coated with type I collagen solution (Beckman Dickinson) by incubating with 2 ml of collagen solution (50 μg/ml in 0.01 N HCl) at room temperature for 1 h. The collagen solution was decanted after incubation. The plate was washed twice with PBS to neutralize residual HCl. Mammary ductal epithelial cells were seeded and grown to confluence. The cell monolayer was then washed twice with PBS and cultured overnight in serum-free growth media. The monolayer was then washed twice with PBS to remove residual serum factors. Treatment media (2 ml) was added to each well. Treatment media consisted of LPS (0, 0.1, 1, 10, 100, 1000 ng/ml) or LPS–rbosCD14 (15 μg/ml) complex formed by pre-incubation overnight at 37 °C. The monolayer was incubated in treatment media for 2, 6 and 24 h. At each time point, the monolayer was washed twice with PBS and lysed in 1 ml of Tri-reagent (Sigma). Total RNA was isolated according to the manufacturer's instructions.

2.6. Interleukin-8 competitive RT–PCR

Competitor molecules for bovine IL-8 and hypoxanthine phosphoribosyltransferase (HPRT) were constructed as previously described (Zarlenga et al., 1995). The first strand of cDNA was synthesized from 1 μg of total RNA at 37 °C for 45 min (Vanden Heuvel et al., 1993). The cDNA reaction contained 67 mM Tris–HCl (pH 8.8, Sigma), 16 mM $(NH_4)_2SO_4$ (Sigma), 0.8 μM EDTA (Sigma), 0.3% β-mercaptoethanol (Biorad), 0.1 mg/ml BSA (Calbiochem, La Jolla, CA), 2.5 mM $MgCl_2$ (Perkin Elmer), 1 mM dNTP (Amersham Pharmacia), 7.5 μg/ml oligo $(dT)_{18}$ (Bioserve, Laurel, MD), 6 units of RNasin (Promega, Madison, WI) and 100 units of MMLV reverse transcriptase (Promega).

The cDNAs were normalized according to their HPRT contents by competitive RT–PCR. The normalized cDNA was PCR amplified with IL-8 competitor. The competitive PCR was run by co-amplification of cDNA and competitor in a reaction containing 67 mM Tris–HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.8 μM EDTA, 0.3% β-mercaptoethanol, 0.1 mg/ml BSA, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.25 μM of each primer and 0.625 units of AmpliTaq polymerase (Perkin Elmer, Branchburg, NJ). The PCR mixture was cycled 30 times at 94 °C for 40 s, 55 °C for 45 s and 72 °C for 1 min. Sequences of HPRT and IL-8 primers are listed in Table 1. The PCR product was separated on a 2% metaphore:GTG (1.8:0.1 wt/wt, FMC) agarose gel and stained with ethidium bromide (Sigma). The intensity of the band was scanned and analyzed by UVP gel documentation system (UVP, Upland, CA). Density ratio between the cDNA and competitor bands was determined and normalized to the HPRT content.

Table 1
Primer sequences for HPRT and IL-8 in competitive RT–PCR

Primer name	Primer sequence
HPRT forward	5′-GGAGATGATCTCTCAACTTTAACTGG-3′
HPRT reverse	5′-CATTATAGTCAAGGGCATATCCCAC-3′
IL-8 forward	5′-GAATTCATGACTTCCAAACTGGCT-GTTGC-3′
IL-8 reverse	5′-TCATGGATCTTGCTTCTCAGCTC-3′

2.7. Intramammary injection of LPS and rbosCD14

Nine clinically normal cows free from intramammary infection and with milk somatic cell counts (MSCCs) <100 000/ml were selected for study. Use of animals for this investigation was approved by the Beltsville Agricultural Research Center and the University of Maryland Animal Care and Use Committees. Quarter foremilk samples were taken aseptically at 12 h and immediately before the morning milking. Right and left rear quarters received 2 ml of LPS (*E. coli* 0111:B4, 0.1 μg/ml) or 2 ml of LPS pre-incubated with rbosCD14 (75 μg/ml) immediately after milking, respectively. The right and left front quarters received 2 ml of 0.8% NaCl or rbosCD14 (75 μg/ml in 0.8% NaCl), respectively. At 12, 24, 36, 48, 60 and 72 h post-injection, quarter milk samples were collected immediately before milking for the determination of total MSCCs using an electronic cell counter (Foss Food Technology, Eden Prairie, MN).

2.8. Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). Data are expressed as the mean ± SE.

3. Results

3.1. Presence of sCD14 in milk

Western blot using anti-ovine CD14 mAb (IgG1) as primary antibody showed that sCD14 was present in skimmed milk with a molecular weight of 46 kDa (Fig. 1). No bands were detected when blots were probed with an unrelated IgG1 mAb or secondary antibody only (data not shown).

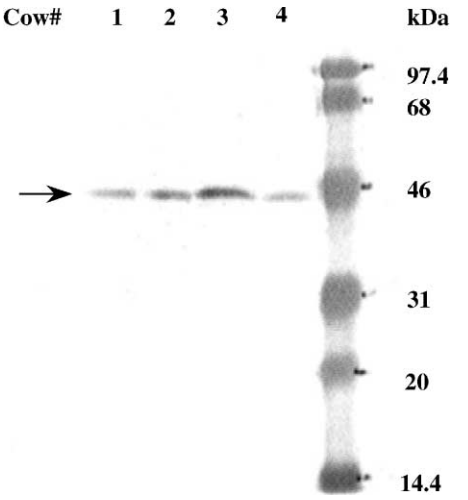

Fig. 1. The presence of bovine sCD14 in whey (arrow). Proteins in whey were separated on a 10% resolving gel, and transferred onto an NC membrane. The NC membrane was probed with anti-ovine CD14 mAb.

3.2. Expression and purification of rbosCD14

Insect cells infected with the recombinant virus containing the N-terminal 358 amino acids of bovine CD14, secreted CD14 into the culture supernatant with the maximal concentration around 96 h post-infection (Fig. 2). The rbosCD14 was purified from culture supernatant using Ni-NTA superflow agarose beads and FPLC system with a typical yield of 4–6 mg/l of culture supernatant (Fig. 3).

3.3. Effect of rbosCD14 on transcription of IL-8 in mammary epithelial cells treated with LPS

Competitive RT–PCR was used to detect changes in transcription of IL-8. Each normalized cDNA was PCR amplified with 50 fg of IL-8 competitor, and

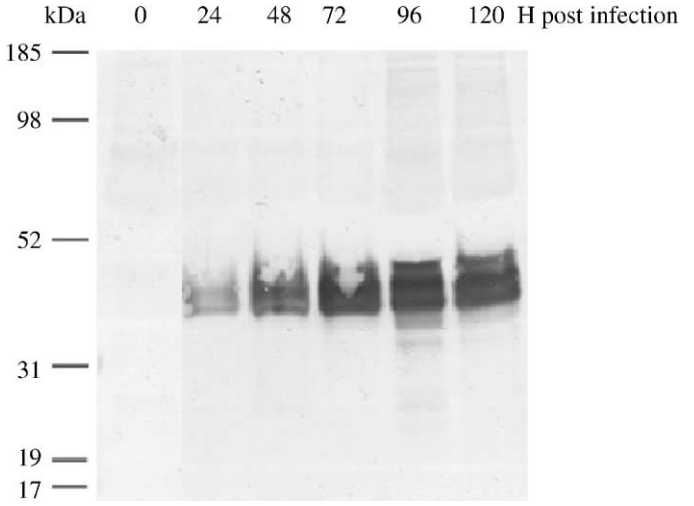

Fig. 2. Expression of rbosCD14 in culture supernatant of infected sf-9 cells at various time points post-infection. Proteins in the culture supernatant (20 μl) for each time point were separated on a 10% Tris–glycine gel under reducing conditions, and were transferred to NC membrane. The membrane was probed with anti-tetra-his-mAb.

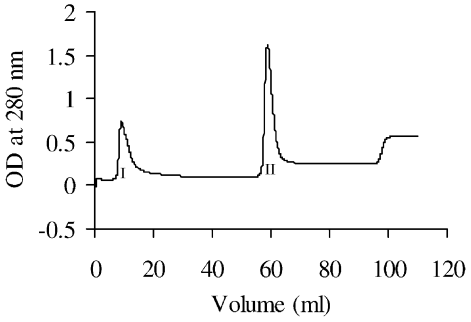

Fig. 3. Purification profile of rbosCD14 using Ni-NTA superflow agarose beads (Qiagen, Valencia, CA) using FPLC. The culture supernatant of infected sf-9 cells was dialyzed against PBS and buffer A, and incubated with Ni-NTA beads in the presence of 10 mM imidazole at room temperature for 3 h. The Ni-NTA beads were packed into a XK16/20 column. The column was washed with 25 mM imidazole (fraction I) and eluted with 100 mM imidazole (fraction II).

the density ratio between cDNA band and competitor band was used as the variable for comparison among samples. Under serum-free condition, cells treated with LPS at 0.1, 1, 10, 100 and 1000 ng/ml for 2 h had a similar density ratio when compared to untreated cells. Increasing treatment time to 6 or 24 h did not change the density ratio. However, cells treated with LPS (1000 ng/ml)–rbosCD14 complex increased the density ratio by 2.2-fold when compared to cells treated with LPS (1000 ng/ml) for 2 h ($P < 0.05$, Fig. 4). As treatment time increased to 6 and 24 h, the LPS–rbosCD14 complexes containing lower concentrations of LPS were able to induce the transcription of IL-8. At 6 h, the complex with 100 ng/ml of LPS increased the density ratio by 24-fold ($P < 0.05$) when compared to LPS alone (Fig. 5). At 24 h, complexes with LPS at 1, 10, 100, 1000 ng/ml increased the density ratio by 11-, 16.8-, 41.6- and 12.5-fold, respectively, when compared to LPS alone at the same concentrations ($P < 0.05$, Fig. 6).

3.4. Effect of rbosCD14 on MSCC in mammary glands injected with low concentration of LPS

Intramammary injection of 0.2 μg of LPS did not induce an increase ($P > 0.05$) in MSCC (Fig. 7). However, injection of LPS–rbosCD14 complex resulted in an increase ($P < 0.05$) in MSCC at 12 and 24 h after injection. Mammary glands injected with either 0.9% NaCl or rbosCD14 did not show an increase ($P > 0.05$) in MSCC during the experimental period.

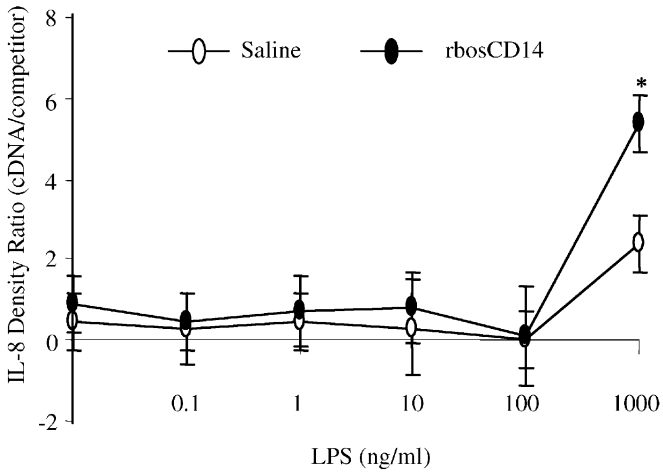

Fig. 4. Effect of rbosCD14 on transcription of IL-8 in mammary ductal epithelial cells treated with LPS for 2 h. Confluent epithelial cells were incubated with LPS (0, 0.1, 1, 10, 100, 1000 ng/ml) or LPS–rbosCD14 (15 μg/ml) complex formed by incubation overnight at 37 °C for 2 h. The density ratio between cDNA band and competitor band after competitive RT–PCR was used to measure changes in transcription of IL-8. Each PCR was run in triplicate. The means and standard errors from three experiments are presented. $*$ denotes that means within the LPS treatment groups differ ($P < 0.05$).

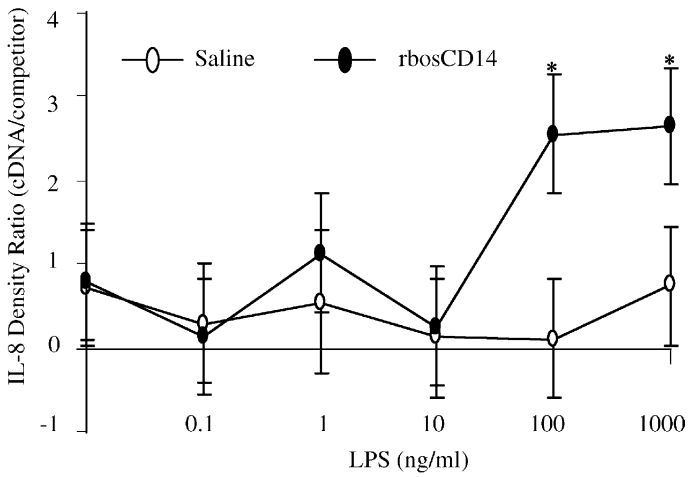

Fig. 5. Effect of rbosCD14 on transcription of IL-8 in mammary ductal epithelial cells treated with LPS for 6 h. Confluent epithelial cells were incubated with LPS (0, 0.1, 1, 10, 100, 1000 ng/ml) or LPS–rbosCD14 (15 μg/ml) complex formed by incubation overnight at 37 °C for 6 h. The density ratio between cDNA band and competitor band after competitive RT–PCR was used to measure changes in transcription of IL-8. Each PCR was run in triplicate. The means and standard errors from three experiments are presented. ∗ denotes that means within the LPS treatment groups differ ($P < 0.05$).

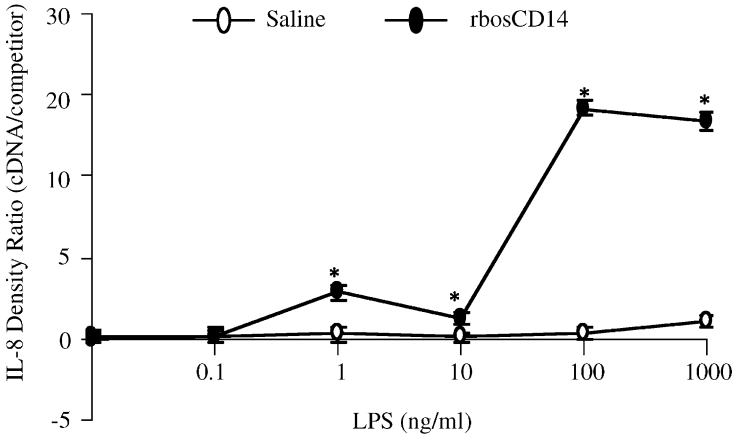

Fig. 6. Effect of rbosCD14 on transcription of IL-8 in mammary ductal epithelial cells treated with LPS for 24 h. Confluent epithelial cells were incubated with LPS (0, 0.1, 1, 10, 100, 1000 ng/ml) or LPS–rbosCD14 (15 μg/ml) complex formed by incubation overnight at 37 °C for 24 h. The density ratio between cDNA band and competitor band after competitive RT–PCR was used to measure changes in transcription of IL-8. Each PCR was run in triplicate. The means and standard errors from three experiments are presented. ∗ denotes that means within the LPS treatment groups differ ($P < 0.05$).

4. Discussion

Previous results from this laboratory indicated that mCD14 is expressed on the cell surface of bovine macrophages and PMN in milk (Paape et al., 1996). In this study, we demonstrated that a 46 kDa protein in milk reacted with anti-ovine CD14 mAb, but not with isotype control mAb. The major source of sCD14 in milk is probably the shedding of mCD14 from cell surfaces of milk macrophages and PMN because

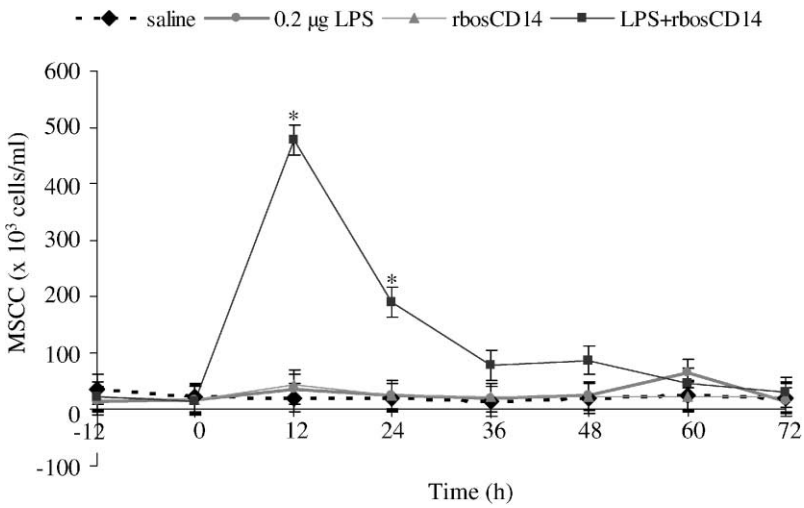

Fig. 7. Changes in MSCCs after intramammary injection. Each of the four mammary glands of each of five cows were injected with either saline, LPS (0.2 μg), rbosCD14 (150 μg) or LPS $(0.2\,\mu g)$ + rbosCD14 (150 μg) at 0 h. Quarter milk samples were collected at −12, 0, 12, 24, 36, 48, 60 and 72 h. The values are the means (±SE) of five cows. ∗ denotes that means within the treatment groups differ ($P < 0.05$).

mammary epithelial cells did not express CD14 as determined by PCR (data not shown). We also detected sCD14 in human milk using anti-human CD14 mAb 60bca (data not shown) by Western blot, which is consistent with others (Labeta et al., 2000; Filipp et al., 2001).

Studies have shown that sCD14 forms a complex with LPS and mediates activation of cells not bearing mCD14 in the presence of low concentrations of LPS (Frey et al., 1992; Pugin et al., 1993). We hypothesized that sCD14 in milk sensitizes the mammary epithelium to low concentrations of LPS. Others reported that recombinant human or bovine sCD14 generated by either transfection of CHO cells or a baculovirus and insect cell expression system were functionally indistinguishable from native sCD14 (Filipp et al., 2001; Tapping and Tobias, 1997). We decide to use rbosCD14, generated by infection of insect sf-9 cells with baculovirus containing C-terminal end truncated boCD14, to test our hypothesis. To eliminate the effect of sCD14 in FBS, we used serum-free conditions by culturing confluent mammary epithelial cells overnight in serum-free media and washing cells before treatment. We choose changes in steady-state levels of IL-8 mRNA (as measured by competitive RT–PCR) as a parameter for mammary epithelial cells responding to LPS because increase in concentrations of IL-8 in foremilk after bacterial challenge corresponds to the acute phase of the inflammatory response when udder swelling, increase in rectal temperature and MSCC, and hypogalactia are most intense. In this study, LPS alone at concentrations of 0.1, 1, 10, 100, 1000 ng/ml did not increase transcription of IL-8 after incubation for 2, 6 and 24 h, when compared to untreated cells. In contrast, LPS–rbosCD14 complex containing 1000 ng/ml induced the transcription of IL-8 after 2 h. By 24 h, LPS–rbosCD14 complex containing 1 ng/ml of LPS was able to induce the transcription of IL-8. These results clearly demonstrated that rbosCD14 sensitized mammary ductal epithelial cells to LPS in vitro.

Others have also reported the expression of cytokine mRNAs for IL-1α, IL-1β, IL-6, IL-10, TNF-α, GM-CSF and IL-8, and secretion of IL-1, IL-6 and IL-8 in bovine mammary epithelial cells after treatment with 1–20 μg/ml of LPS (Okada et al., 1997, 1999; Boudjellab et al., 1998) in the presence of serum. This study was the first that defined the role of sCD14 in the response of bovine mammary epithelial cells to LPS.

Leukocyte recruitment from blood into the mammary gland is essential for host defense against intramammary infections. Total and differential MSCC are used as diagnostic tools in mastitis research (Paape et al., 2000). We further tested our hypothesis by

injecting LPS into the mammary gland and measuring changes in MSCC. We used a low concentration of LPS that would not increase MSCC. However, intramammary injection of LPS–rbosCD14 containing the same concentration of LPS increased MSCC 12 and 24 h after injection. These results indicated that rbosCD14 sensitized mammary gland to LPS in vivo. The sensitization of the mammary gland to LPS should be beneficial to the host because a faster recruitment of leukocytes may be induced when just a few bacteria are present in the mammary gland. Studies using LBP-deficient mice have shown that defects in CD14-dependent cellular response to LPS protected mice from a lethal challenge with LPS, but prevented bacterial clearance after bacterial challenge in vivo (Jack et al., 1997; Wurfel et al., 1997). A delay in leukocyte recruitment after intramammary coliform infections results in approximately 10 times more bacteria (Erskine et al., 1989). These studies emphasize the importance of a rapid and early inflammatory reaction in protecting the host from an overwhelming bacterial infections.

Variation in the sensitivity of cows to LPS was observed during this study. We reported results from cows that did not have an increase in MSCC after injection of 0.2 μg of LPS. Mammary glands of four out of nine cows had an increase in MSCC after infusion with 0.2 μg of LPS. Of the five cows who did not respond to 0.2 μg of LPS an increase in MSCC after injection of LPS–rbosCD14 complex containing 0.2 μg of LPS was observed. Variation in the kinetics of the MSCC increase after LPS or *E. coli* injection was also reported by others (Erskine et al., 1989).

We proposed that interaction of ductal epithelium with LPS may provide initial mediators for an inflammatory response against gram-negative bacteria in the mammary gland. This study provided evidence that mammary ductal epithelial cells are able to sense low concentrations of LPS in the presence of sCD14 in vitro and in vivo. Further experiments that determine correlations among mammary gland sensitivity to LPS, MSCC and incidence of coliform mastitis after experimental exposure to *E. coli* will facilitate the understanding of the initiation of inflammatory response in the bovine mammary gland, and in designing preventive and therapeutic regimens for controlling coliform mastitis.

5. Conclusions

Recombinant bovine CD14 (rbosCD14) was produced using sf-9 cells and a recombinant virus containing the N-terminal 358 amino acids of bovine CD14. Complexes of LPS–rbosCD14 induced IL-8 mRNA expression in mammary ductal epithelial cells. Intramammary injection of LPS–rbosCD14 complex induced diapedesis of leukocytes. In normal mammary quarters, a 46 kDa protein in milk whey reacted with anti-ovine CD14 mAb. Endogenous sCD14 in milk may be important in initiating host responses to gram-negative bacterial infections.

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